

BASIC RESEARCH

Periosteum as a source of mesenchymal stem cells: the effects of TGF- β 3 on chondrogenesis

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INTRODUCTION: Numerous experimental efforts have been undertaken to induce the healing of lesions within articular cartilage by re-establishing competent repair tissue. Adult mesenchymal stem cells have attracted attention as a source of cells for cartilage tissue engineering. The purpose of this study was to investigate chondrogenesis employing periosteal mesenchymal cells.

METHODS: Periosteum was harvested from patients who underwent orthopedic surgeries. Mesenchymal stem cells were characterized through flow cytometry using specific antibodies. The stem cells were divided into four groups. Two groups were stimulated with transforming growth factor β 3 (TGF- β 3), of which one group was cultivated in a monolayer culture and the other was cultured in a micromass culture. The remaining two groups were cultivated in monolayer or micromass cultures in the absence of TGF- β 3. Cell differentiation was verified through quantitative reverse transcription-polymerase chain reaction (RT-PCR) and using western blot analysis.

RESULT: In the groups cultured without TGF- β 3, only the cells maintained in the micromass culture expressed type II collagen. Both the monolayer and the micromass groups that were stimulated with TGF- β 3 expressed type II collagen, which was observed in both quantitative RT-PCR and western blot analysis. The expression of type II collagen was significantly greater in the micromass system than in the monolayer system.

CONCLUSION: The results of this study demonstrate that the interactions between the cells in the micromass culture system can regulate the proliferation and differentiation of periosteal mesenchymal cells during chondrogenesis and that this effect is enhanced by TGF- β 3.

KEYWORDS: Periosteum; Mesenchymal Stem Cells; Chondrogenesis; High-density Culture; Monolayer Culture.

Mara CS, Sartori AR, Duarte ASS, Andrade ALL, Pedro MAC, Coimbra Ibsen B. Periosteum as a source of mesenchymal stem cells: the effects of tgfb- β 3 on chondrogenesis. *Clinics*. 2011;66(3):487-492.

Received for publication on October 9, 2010; First review completed on November 9, 2010; Accepted for publication on December 6, 2010

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INTRODUCTION

Articular cartilage exhibits low capacity for self-repair after joint damage. Previous research has shown that damaged cartilage tissue shows limited potential for repair or regeneration due to its avascularity and due to the presence of relatively few chondrocytes that exhibit low mitotic activity.¹ In the instance of full-thickness articular cartilage defects that penetrate the cartilage tissue, the repair of hyaline cartilage is often performed under restrictive conditions that are frequently insufficient for complete repair. Articular cartilage defects correlate with pain and joint dysfunction and remain a practical problem, particularly in younger patients.

Articular cartilage is a connective tissue, which performs highly specialized functions that are adapted to the local

needs of the site. In native articular cartilage, the extracellular matrix (ECM) is primarily composed of a network-like structure of proteoglycans and type II collagen among other proteins. Chondrocytes are the cellular component of the cartilage and are responsible for the production and degradation of the ECM.²

Mesenchymal stem cells (MSCs) are undifferentiated pluripotent cells that are capable of differentiating into many cell types. Adult human MSCs have been derived from a variety of tissues and have exhibited the potential of participating in the growth and repair processes. MSCs are an attractive source of cells for regenerative medicine because they can be harvested in a minimally invasive manner, isolated easily, and expanded without difficulty while preserving their pluripotent capacities including the capacity for chondrogenesis. Therefore, MSCs may be a suitable autogenous cell source for the repair of articular cartilage.^{3,4} Also, factors such as TGF play critical roles in the compaction and shaping of groups of mesenchymal cells.⁵

Various cell-based therapies have been evaluated for the treatment of defects in the articular cartilage. Autologous

chondrocyte implantation (ACI) is a biological method for the treatment of large, full-thickness chondral defects of the knee. Briefly, ACI involves the implantation of a suspension of cultured autologous chondrocytes beneath a tightly sealed periosteal flap. Periosteal coverage is a popular technique for repairing cells or cell/scaffold composites in cartilage defects.⁶ This method also implants a large quantity of progenitor cells, which differentiate into chondrocytes.⁷⁻⁹ However, a previous study found that the transplantation of MSCs or chondrocytes without a periosteal patch would be advantageous because periosteum may be associated with hypertrophy or ossification.¹⁰ On the other hand, Brittberg et al.⁶ reported that transplantation using autologous chondrocytes produced excellent results in patients with patellar defects. However, this approach was limited by the number of acquired chondrocytes and their proliferation rate. Moreover, it has been well characterized that chondrocytes cultured in a monolayer for a period of more than two weeks undergo de-differentiation.^{11,12}

N-cadherin, a cell-dependent adhesion molecule, is strongly expressed in condensed mesenchyme and has demonstrated a role in chondrogenesis in murine cell lines.¹³⁻¹⁵ Therefore, we investigated the activity of periosteum-progenitor mesenchymal cells (PPMCs) in high-density micromass cultures. There are currently no published reports on the use of this technique in human cells.

The successful employment of cell therapy for the treatment of cartilage injury requires the determination of the conditions or parameters that are necessary for the selection of an appropriate cell. In the present study, our aim was to evaluate the ability of PPMC to differentiate into chondrocytes and express collagen type II by comparing high-density micromass cultures with monolayer cultures in the presence and absence of TGF- β 3.

MATERIALS AND METHODS

Characterization of periosteum-progenitor mesenchymal stem cells

The periosteum samples were harvested from the proximal tibial tissues of four human donors of various ages (ranging from 40-60 years) who underwent surgical knee replacement procedures. According to the protocol described by Jansan et al.,¹⁶ the tissue samples were rinsed twice with phosphate-buffered saline (PBS) containing an antibiotic-antimycotic solution (Sigma, USA). The samples were minced into small slices and treated with 1% collagenase solution (Sigma, St. Louis, MO, USA) in PBS for digestion at 37°C for 20 min. The collagenase solution was drained and the periosteum-derived cells were collected after washing with PBS. The cell suspension was centrifuged at 1200 RPM, and the cell pellet was re-suspended in low-glucose Dulbecco's modified Eagle's medium (DMEM-Gibco®) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution at 37°C for one week in a chamber containing 95% humidified air and 5% CO₂. When the cells reached 80% confluence, they were trypsinized (5 mg trypsin/ml PBS), washed in PBS, re-suspended in 20 ml medium, and replated into 75-cm² bottles (Cellstar®) for expansion. After three passages, the cells were trypsinized again and analyzed using flow cytometry (FACSort, BD, San Jose, CA, USA). The following monoclonal antibodies were used: CD90-PECY5, CD 105-PE, CD 29-FITC, CD73-PE, STRO-PE, CD34-PE, CD 45-SPRD and HLA DR-FITC. These antibodies were

employed because they are markers of mesenchymal progenitor cells, which do not express the typical hematopoietic antigens.¹⁷

For immunophenotypic characterization, MSCs at the third passage were trypsinized, harvested, washed once with PBS, and re-suspended in PBS. Cells (1×10^5 per sample) were stained at room temperature for 30 min with isotype control mAbs or with specific anti-human antibodies. These samples were mixed gently and incubated for 20 min at 4°C in the dark, then washed and re-suspended in staining buffer (PBS-BSA). Data from 10,000 events were recorded, and flow cytometry was used to measure the binding of antibodies (CD90 PECY5, HLA DR FITC CD34 PE) relative to that of isotype-matched control antibodies. The specific fluorescence labeling was analyzed using a FACSCalibur flow-cytometry instrument (Becton Dickinson, USA) using the Cell Quest software (BD Bioscience, San Jose, CA, USA).

Chondrogenic Differentiation

After expansion, the mesenchymal cells were re-suspended in a chondrogenic culture medium consisting of high-glucose Dulbecco's modified Eagle's medium (Gibco), Invitrogen, California, USA, 10 ng/ml TGF- β 3 (R&D Systems), 100 nM dexamethasone, 1×10^{-8} M ITS+1 premix, 40 μ g/ml proline, and 25 μ g/ml ascorbate-2-phosphate (all obtained from Sigma-Aldrich, Poole, U.K., <http://www.sigmaaldrich.com>).¹⁸⁻²⁰ To achieve chondrogenesis in high-density cell culture, micromass-culture cells were seeded at a density of 5×10^5 cells per 100 μ l of medium onto dry wells in a 96-well plate (Corning Life®). After two hours, the wells were slowly filled with 0.2 ml of chondrogenic medium in which the cells were maintained for 21 days at 37°C in 5% CO₂ and 95% air. The medium was refreshed every three to four days. A second model of culture (i.e., the monolayer system) was applied by seeding cells into Costar six-well cell-culture plates (Corning) at a density of 1×10^6 cells/cm². The medium in this system was also changed every three to four days.

RNA Isolation, Reverse Transcription and RT-PCR

Total mRNA was extracted and prepared using the TRIZOL reagent according to the manufacturer's instructions (InvitrogenTM Life Technologies, Carlsbad, CA92008, USA). Total RNA (1 μ g) was treated with 1 U of deoxyribonuclease I (DNase I) (InvitrogenTM Life Technologies, Carlsbad, CA92008, USA) to digest any contaminating genomic DNA. Reverse transcription was performed using SuperScriptII (Invitrogen) according to the manufacturer's protocol. Quantitative PCR was conducted using SYBR green (applied quantitative real-time PCR was performed on a 96-well-plate ABI Prism 7000 Sequence Detection machine; Applied Biosystems, Foster City, CA) from the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The total volume (12 μ l) of each PCR reaction contained 6 μ l SYBR Green PCR Master Mix, 10 ng cDNA (3 μ l), and 150 pM (3 μ l) of each of the forward and reverse primers. The real-time PCR reaction was carried out at 95°C for 10 min (activation), 40 cycles of 95°C for 15 sec, 60°C for 20 sec, 72°C for 20 sec (amplification), and 72°C for 1 min (final extension). The melting curves were acquired after the Polymerase Chain Reaction (PCR) to confirm the specificity of the amplified products. A standard curve, based on the threshold values of the cycle, was used to evaluate gene expression. Glyceraldehyde 3

Table 1 - The primer sequences used in qRT-PCR. Primer sequences for all genes were designed using the ABI Primer Express program (Applied Biosystems, Foster City, CA, USA).

Collagen II Forward	GGCAATAGCAGGTTACGTACA
Collagen II Reverse	CGATAACAGTCTTGCCCACTT
Aggrecan Forward	TCGAGGACAGCGAGGCC
Aggrecan Reverse	TCGAGGGTGTAGCGTAGAGA
GAPDH Forward	GCACCGTCAAGGCTGAGAAC
GAPDH Reverse	CCACTTGATTTGGAGGGATCT

phosphate dehydrogenase (GAPDH) was employed as an internal control.

Primer Sequences

The primer sequences for all genes were designed using the ABI Primer Express program (Applied Biosystems, Foster City, CA, USA). The specific primers that were used are listed in Table 1.

Western Blot Analysis

The proteins secreted by the cells into the culture medium were analyzed as follows. The proteins were precipitated using 2 mg/ml of pepsin (Sigma-Aldrich®) and 30 µl/ml of acetic acid, glacial P.A. The samples were maintained in 30°C for 30 min and then stored overnight at 4°C on a shaker. Next, the samples were centrifuged for 90 min at 6400 RPM. Finally, the pellets were washed twice with PBS buffer, and the quantity of protein was determined using a spectrophotometer. Next, 30 µg of protein was separated on a 10% SDS-PAGE (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis gel and transferred onto a nitrocellulose Hybond membrane (Amersham) using trans-blot apparatus (Invitrogen). The membranes were blocked with 5% (v/v) skimmed milk in PBS containing 0.1% Tween 20. Finally, the blot was sequentially incubated with a 1:2000 diluted polyclonal type II collagen antibody (Chemicon®) capable of recognizing the C-terminal telopeptides of human type II collagen, which has a molecular weight of 70 kD (21). The antibody was rinsed three times with PBS containing 0.1% Tween 20 and then incubated with a peroxidase-conjugated secondary antibody against rabbit IgG. The signals were visualized using an ECL kit (Amersham Biosciences UK Limited Chalfont Buckinghamshire, England).

Statistical Analysis

Statistical analysis was performed using the one-way analysis of variance (ANOVA) test with commercially available software (InStat; GraphPad Software, San Diego, CA, USA, <http://www.graphpad.com>). The confidence interval was 95%. Considering the multiple comparisons between groups, *P* values below 0.05 were defined as significant.

RESULTS

The MSC which firmly attached to the surface of the cell-culture plate, formed colonies, and presented spindle-shaped or fibroblast-like morphology and agranular appearance. The sub-cultured cells grew in a monolayer and attained a stable, fibroblast-like morphology with no signs of granulation (Figure 1).

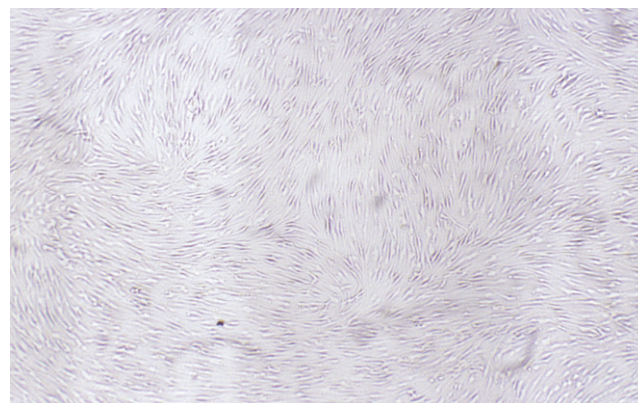


Figure 1 - The morphology of PPMCs. The cells exhibited a fibroblastoid morphology in a monolayer and then attained a stable, fibroblast-like morphology with no signs of granulation.

The expanded cells exhibited cell-surface antigens that are typically associated with mesenchymal stem and progenitor cells. The results of immunophenotypic characterization of the PPMCs (where the percentage of cells that were positive for the cell-surface markers was determined using flow cytometry) indicated that the cells were homogeneously positive for the following antigens: CD90 PE: 77.29%, CD105 PE: 94.89%, CD29 FITC: 93.33%, CD73: 92.47%, STRO1 PE: 31.41%, CD34 PE: 3.11%, CD45 S PRD: 4.63%, HLA-DRFITC: 0.53% (Figure 2).

RT – PCR

After three weeks, the levels of type II collagen and aggrecan mRNA were significantly higher upon chondrogenic induction compared to the control group (i.e., monolayer culture without TGF-β3) (*P*<0.001). The expression of both genes, type II collagen and aggrecan, was highest in the cells that were cultured in the micromass system in the presence of TGF-β3 (*P*<0.001). The cells that were cultured in the micromass system without TGF-β3 were not statistically different from the cells that were cultured in the monolayer system with TGF-β3 (*P*>0.05). The cells that were cultured in the monolayer system without TGF-β3 exhibited no expression of type II collagen (Figure 3).

Western Blot

The expression of type II collagen in cultured cells was also greater in the cells that were cultured with TGF-β3 compared to the cells that were cultured without TGF-β3. Micromass cultures that were cultivated without TGF-β3 also exhibited higher type II collagen expression compared to the monolayer cultures cultivated with TGF-β3, corroborating the real-time PCR results. The cells that were cultured in a monolayer without TGF-β3 did not express type II collagen (Figure 3).

DISCUSSION

Articular cartilage shows limited capacity for self-repair after joint damage. The regeneration of articular cartilage using cell-based therapies requires the identification of available cells that have the ability to differentiate into chondrogenic cells. Several clinical studies have investigated methods such as abrasion arthroplasty,²² microfrac-

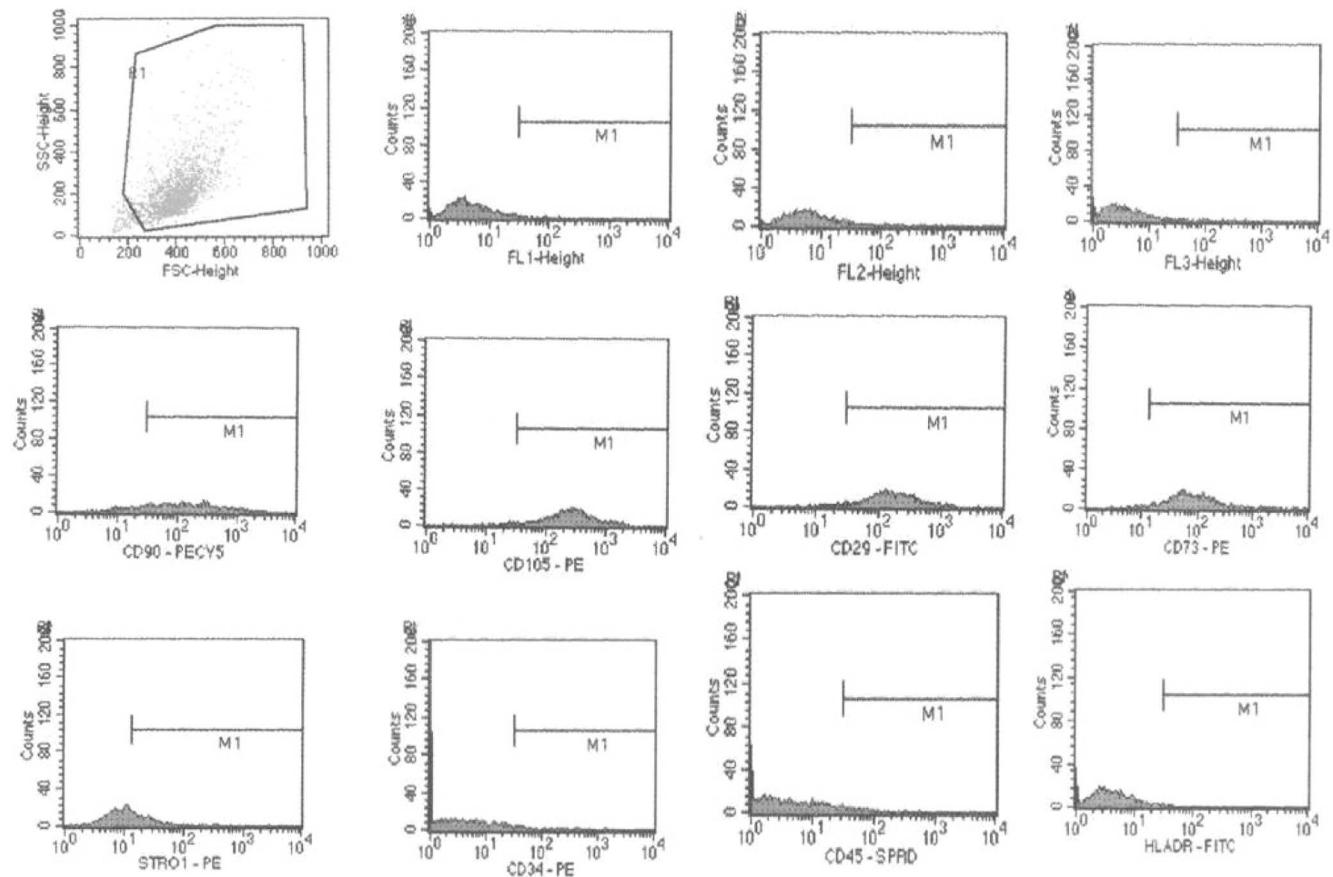


Figure 2 - The expanded cells exhibited cell-surface antigens that are typical of mesenchymal stem and progenitor cells. The results of the immunophenotypic characterization of the PPMCs indicated that the cells were homogeneously positive for the following antigens: CD90 PE: 77.29%, CD105 PE: 94.89%, CD29 FITC: 93.33%, CD73: 92.47%, STRO1 PE: 31.41%, CD34 PE: 3.11%, CD45 S PRD: 4.63% and HLADR – FITC: 0.53%.

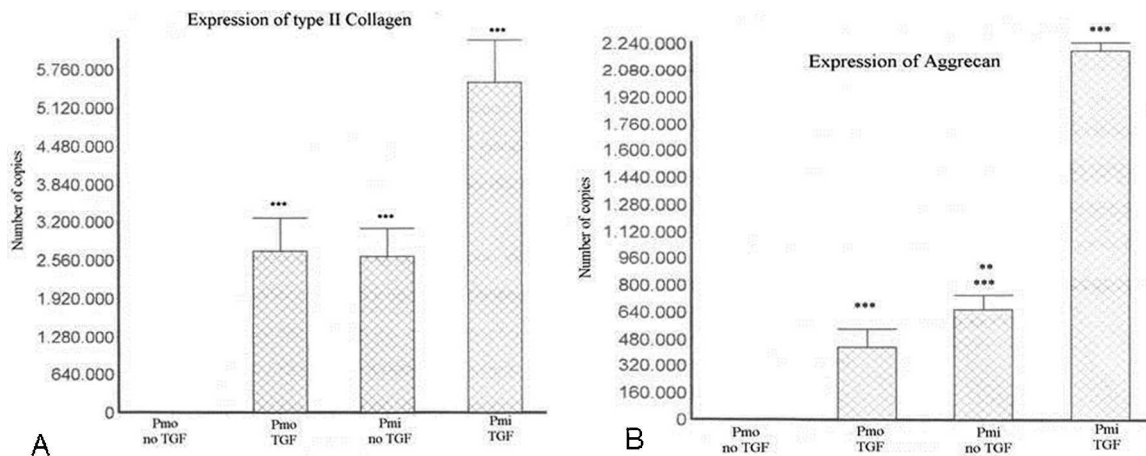


Figure 3A - The expression of type II collagen. RT-PCR analysis was conducted after three weeks of culturing of the periosteum-derived cells in monolayer and micromass systems. We observed that in the micromass culture, the addition of TGF- β 3 (P mo TGF and P mi TGF) increased the expression of type II collagen ($P < 0.001$). No statistical difference was observed between cells grown in a monolayer in the presence of TGF β 3 (P mo TGF) and cells grown in a micromass without TGF- β 3 (P mi no TGF) ($P > 0.05$). **B.** The expression of aggrecan. RT-PCR analysis was conducted after three weeks of culturing of the periosteum-derived cells in both monolayer and micromass systems. We observed that in the micromass culture, the cells grown in the presence of TGF β - β 3 exhibited increased expression of Aggrecan ($P < 0.001$) compared to the cells grown in the monolayer with TGF- β 3 (P mi TGF and P mo TGF). The cells in the micromass culture without TGF- β 3 (P mi no TGF) expressed less aggrecan compared to the cells in the monolayer culture with TGF- β 3 (P mo TGF), ($P < 0.01$).

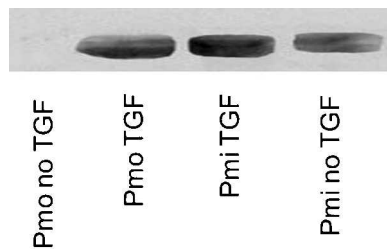


Figure 4 - The expression of type II collagen in the culture was greater in cells that were treated with TGF- β 3 than in cells cultured without TGF- β 3. Micromass cultures without TGF- β 3 also exhibited greater type II collagen expression compared to the monolayer cultures with TGF- β 3, corroborating the real-time PCR results. The cells that were grown in the monolayer without TGF- β 3 did not express type II collagen.

ture chondroplasty,²³ osteochondral grafting²⁴⁻³⁰ and the transplantation of autologous chondrocytes into the defects. Currently, the autologous chondrocyte implant (ACI)⁶ is the most widely employed surgical procedure for effecting the regeneration of articular cartilage. However, this procedure remains somewhat risky because chondrocytes in suspension can leak from the graft site after load-bearing activity is resumed. Moreover, long-term studies have reported that grafts can produce fibrocartilage.³¹ The origins of the cells employed by this technique remain unclear (i.e., we do not know whether the cells originate from the patient's chondrocytes or from the periosteal tissue) because studies have shown that periosteum contains cells that are capable of differentiating into chondrocytes.^{32,33} In this study, we induced chondrogenesis by culturing MSCs derived from periosteum in a micromass system in the presence of TGF- β 3. We optimized culture conditions for chondrogenesis through high-density micromass plating and TGF- β 3 treatment. A previous study concluded that the use of periosteum coverage for retaining chondrocytes within an osteochondral defect exhibited no advantage compared to a type I/III collagen membrane. However, such studies demonstrated no statistical difference in the outcome with regard to arthroscopic findings of both clinical and functional assessment after one year.³² Sub-periosteal injection of TGF- β 3 has been known to stimulate the proliferation of periosteal mesenchymal cells to induce chondrogenic differentiation³⁴⁻³⁶ as well as mediate differentiation into other mesenchymal cell types.³⁷ Thus, the aim of our study was to determine the effect of TGF- β 3 on PPMCs in high-density micromass cultures compared to those in monolayer systems. Previous studies have demonstrated the capacity of PPMCs to cause chondrogenesis.¹⁶ In our study, we also found that type II collagen mRNA was expressed more robustly in a micromass culture system than in a monolayer system following treatment with TGF- β 3. Moreover, we also found that cells kept in a micromass culture system produce type II collagen even in the absence of TGF- β 3. We attributed this finding to the activity of adhesion molecules, which play a pivotal role in chondrogenesis. As other researchers have demonstrated,³⁸ these molecules are upregulated when the cells are grown in micromass cultures. Specifically, in high-density micromass cultures, these cells express the adhesion molecule N-cadherin, which plays an important role in the regulation of the chondrocytic phenotype.^{14,15,39} Cellular condensation is a required early step in the initiation of mesenchymal

chondrogenesis because this condensation is accompanied by the elevated expression of N-cadherin *in vitro*. High cell density has also been reported as a requirement for mesenchymal chondrogenesis.⁴⁰ Our results suggest that the *in vitro* pretreatment of micromass cultures with TGF- β 3 increases the ability of periosteum to undergo chondrogenesis and produce hyaline cartilage. Our findings also suggest that periosteum can be used as a source of chondrocytes for autologous implants because the periosteum cover provides cells for the repair of the lesion. Moreover, periosteum-derived chondrocytes can be used for the generation of new transplants by being applied to a different scaffold. Further studies using periosteal chondrocytes grown in micromass cultures are required to evaluate whether the chondrocytes maintain their phenotypes in animal models of chondral defects.

ACKNOWLEDGMENTS

Source of support: This study was supported by the CNPq (Conselho Nacional de Desenvolvimento e Pesquisa).

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